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INVESTIGATION OF THE ANTIBACTERIAL ACTIVITY, ANTIBIOFILM PROPERTIES AND PHYTOCHEMICAL COMPOSITION OF POLY-HERBAL EXTRACTS OF PERESKIA ACULEATA, TRACHYSPERMUM AND SPHAGNETICOLA TRILOBATA

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Abstract:
The global problem of increasing resistance to antibiotics has prompted researchers to seek out new antimicrobial agents derived from natural compounds. The objective of the present investigation was to assess the antibacterial efficacy of commonly used but unexplored polyherbal formulations in the context of wound recovery treatment. A total of 3 kinds of plants were combined in varied quantities to generate 11 polyherbal concoctions. Various solvents were used to extract the constituents of 11 polyherbal concoctions. The extraction process involved the use of cold maceration after collecting and grinding the plant materials. The antibacterial activities of the polyherbal extracts were tested using the technique of diffusion on agar wells at a dosage of 30 mg/ml. The MIC (minimum inhibitory concentration) was estimated using the sequential dilution method. The polyherbal concoction (G) had the greatest efficacy against *S. aureus* (37±1.0 mm), *E. coli* (29±1.5mm), and *B. subtilis* (35±0.40 mm). The lowest inhibitory concentration was also assessed using a ratio of 1:1:2:1, consisting of *Pereskia aculeata, Sphagneticola trilobata* leaves, and *Trachyspermum ammi* seeds and leaves. The minimum inhibitory concentration (MIC) values against *E. coli, S. aureus* and *B. subtilis* were determined to be 50±0.12 mg/mL, 70±0.17 mg/mL, and 30±0.20 mg/mL, correspondingly. The antibacterial efficacy of polyherbal recipe G is enhanced, surpassing that of other examined recipes. This recipe warrants further scientific scrutiny for additional validation.

Keywords: *Polyherbal, Pereskia aculeata leaves, Trachyspermum ammi seeds,* and *Sphagneticola trilobata leaves.*

1. Introduction

Antibiotics are a crucial tool in combating bacterial illnesses and have significantly improved the standard of human life in terms of wellness since their inception. Nevertheless, in recent decades, the efficacy of numerous widely prescribed antibiotics has diminished, posing a significant risk to the aforementioned health advantages [1, 2]. This decline can be attributed not solely to the occurrence of adverse effects associated with many antibiotics but additionally to the creation of antibiotic-resistant bacteria [3]. Patients infected with bacteria that resist antibiotics in a wound may experience prolonged illness and require more expensive treatment [4]. Therefore, effectively caring individuals with wound infections necessitates heightened

attention to prevent the emergence of resistance. The primary determinant impacting the emergence of tolerance is the utilization of antibiotics [5].

In order to prescribe antibiotics correctly, it is vital to have knowledge about the necessary conditions for wound infection, the microbes that cause them, and their current variations in susceptibility [6]. Given the established effectiveness of wound healing and wound irrigation, the first treatment approach for infected wounds should typically not involve providing antibacterial agents [7, 8]. Systemic antibacterial agents should only be used when there is evidence of infection extending through the subcutaneous soft tissues, or in instances of ascending limb infection or severe sepsis. In order to reduce the impact of specific antibacterial medications on the natural microorganisms found on the skin and in the digestive system, it is advisable to choose antibiotics that target a limited range of bacteria [9].

Systemic antibiotic treatment should be adjusted empirically, taking into account the findings of wound cultures. Topical antibacterial agents have shown effective in treating patients with wounds that are infected [10]. Establishing criteria for the judicious utilisation of systemically and topical antimicrobials is a crucial strategy to restrict and manage the emergence of resistance. Exploring novel pharmaceuticals with reduced resistance is crucial [11]. Antibiotics are organic compounds, either natural or manmade, that have the ability to effectively combat germs [12].

Antimicrobial resistance (AMR) refers to the capacity of bacteria to endure and remain viable when exposed to antimicrobial agents. Different categories of antimicrobial compounds, including antibiotics, disinfecting agents, and food preservation agents, can be utilised to combat microorganisms by diminishing their ability to proliferate, impeding their multiplication, or even causing their demise [13]. The bacteria developed antimicrobial resistance (AMR) as a result of the long-term and widespread usage of antibiotics [14]. Owing to the indiscriminate utilisation of antimicrobial agents, bacteria have acquired resistance to a wide range of antibiotics. The management of infectious disorders has been greatly complicated by this issue [15]. Antimicrobial resistance (AMR) has become a significant worry due to its impact on mortality rates and economic costs [16]. Organisms that were exposed to the initial commercially manufactured antibiotics developed significant resistance to individual drugs. An enzyme called penicillinase, identified by Hausner et al., is responsible for the degradation of the antibiotic penicillin, leading to resistance among staphylococci [17].

Multidrug resistance is a form of resistance that bacteria develop by many ways, such as altering their membranes to either decrease the uptake of drugs or increase their efflux. Other strategies include inactivating drugs or modifying them chemically [18]. The widespread transmission of bacterial illnesses that are resistant to several drugs is a major worry in terms of therapy. Gram-negative bacteria are a type of multidrug-resistant (MDR) bacteria that hinder the efficacy of numerous clinically utilised antibiotics [19]. The primary mechanism responsible for multidrug resistance (MDR) in gram-negative bacteria involves alterations in membrane permeability, namely through the reduction of passive antibiotic uptake or the enhancement of active efflux to expel the antibiotic [20]. Multi-drug resistant (MDR) bacteria exhibit the ability to last for extended periods, proliferate under conditions of limited nourishment, and possess the capacity to inhabit damaged skin. This poses a substantial risk to public health worldwide [21].

Traditional or herbal medicines refer to remedies derived from plants and are generally considered safe at the recommended dosage, as they have been historically used in many cultures [22]. Plants continue to be the most plentiful natural primary origin of active medications and are quite beneficial in the ethnomedical management of many illnesses [23]. Throughout ages, plants have been utilized for the treatment of infectious diseases and are widely recognized as a significant reservoir of novel antibacterial substances [24]. Multiple studies have been conducted to investigate the antibacterial properties of herbal plant extracts

derived from various parts such as roots, stems, leaves, or flowers [25]. Therapeutic efficacy is asserted for the treatment of jaundice, cough, chronic ulcers and wounds, diarrhoea, dysentery, leprosy, piles, skin disorders, chronic bronchitis, syphilis, typhoid, and whooping cough. The prevalence of cough, genito-urinary disorders, sore throat, and fevers among traditional herbalists in India has sparked our curiosity to scientifically study these herbal remedies [26]. The objective of this study was to assess the antibacterial efficacy of certain medicinal plants commonly employed in Ayurvedic and traditional medicine for the management of symptoms resulting from microbial infections. Hence, samples derived from the subsequent four plants belonging to distinct botanical families were examined to assess their potential efficacy against bacterial pathogens: leaves of *Pereskia aculeata* and *Sphagneticola trilobata leaves,* and *Trachyspermum ammi seed and leaves.*

2. Material and methods:

2.1.Sample collection and preparation:

1kg of *Pereskia aculeata* leaves were harvested directly from the garden. The weight and moisture content of the petals were determined to be constant at 2-3% after drying them in the shade and using a hot air oven at 50ºC. The seeds and leaves of *Trachyspermum ammi* and as well as *Sphagneticola trilobata* leaves were obtained from the market. The fresh leaves and seed were meticulously washed using a continuous flow of tap water. The washed peels were subsequently dehydrated at a temperature of 50ºC for a duration of forty-eight hours in a hot air oven, and then let to dry naturally for a period of two days. The seed and leaves were crushed into small fragments measuring around 1×1 cm. These fragments were then ground into a powder and combined in various proportions to evaluate their effectiveness in inhibiting the growth of microorganisms [27].

2.2.Preparation of extracts:

The leaves of *Pereskia aculeata, Sphagneticola trilobata,* and *Trachyspermum ammi* seed and leaves are taken in equal amounts of 30g each, as specified in Table 1. The extraction process is then conducted using the solvent evaporation method. The process was iterated incessantly to achieve a thorough and comprehensive extraction of crude. The resulting extract was evaporated to a dry residue under decreased pressure at the ambient temperature. The condensed residue was preserved at a temperature of 4 degrees Celsius and employed for subsequent investigation [28].

Table 1: Mixture of powdered herbs carefully picked and prepared.

S. No	**Solvent**	**(*P. aculeata*: *T. ammi (L.)*: *T. ammi (s)* : *S. trilobata*)**
1	Distilled water	1: 1: 1: 1 (A)
2	Methanol (50%)	1: 1: 1: 1 (B)
3	P. ether (50%)	1: 2: 1 : 1 (C)
4	Chloroform (50%)	1: 2 : 1 : 1 (D)
5	Methanol (50%)	1: 1: 2 : 1(E)
6	P. ether (50%)	1: 1 : 2 : 1 (F)
7	Methanol (50%)	1: 1 : 1 : 2 (G)
8	Chloroform (50%)	1: 1 : 1 : 2 (H)
9	P. ether (50%)	2: 1 : 1 : 1 (I)
10	Chloroform (50%)	2: 1 : 1 : 1 (J)

11	Methanol (50%)	1: 3 : 1 : 2 (K)

2.3.Antibacterial activities of polyherbal mixtures.

A test for antibiotic sensitivity was conducted to assess the effectiveness of the antimicrobial action. Three Petri plates were prepared with 45 ml of nutrient agar material. The equipment underwent autoclaving at a temperature of 121°C for a duration of 30 minutes. Following autoclaving, 15 ml of this material was aseptically placed into Petri plates and allowed to solidify for approximately 10 minutes. *B. subtilis, Staphylococcus aureus, and Escherichia coli,* were evenly distributed around the plate. Wells were made on every plate. The samples were loaded with 40 microliters. To prevent any form of contamination, the entire procedure was conducted under the laminar flow hood. The plates were incubated at a temperature of 37º C for a duration of 24 hours in an incubator. The measurement of zones of inhibition was conducted in millimeters, as reported by Sharma et al. in 2021 [29].

The minimal inhibitory concentration (MIC) refers to the lowest concentration of an antimicrobial agent that is capable of preventing the observable development of bacteria following an overnight incubation period. A lower minimum inhibitory concentration (MIC) signifies superior antibacterial and antifungal efficacy. A nutrient broth solution was made by dissolving 0.26 grammes of nutrients in each of the 6 test tubes.3 microliters of nutritional broth were put into each test tube. The equipment underwent autoclaving at a temperature of 121°C for a duration of 30 minutes. Following autoclaving, a 100-microliter sample was sequentially placed in the first through sixth test tubes, with 100 microliters being removed from the sixth test tube. Injected 20 microliters of the bacterial sample into all test tubes, excluding the 6th test tube. The test tubes were incubated in a shaker incubator at a temperature of 37° C for a duration of 24 hours, except for the 6th test tube. Stored the sixth test tube in the refrigerator at a temperature of 4 degrees Celsius. The optical density at 600nm was measured after a 24-hour period. The source cited is a publication by Sharma et al. in 2021 [29].

2.4. Biofilm formation:

In summary, a culture medium called tryptic soy broth (TSB) was prepared with the addition of 2% (w/w) d-glucose (TSBGlc). Well-isolated colonies that were grown overnight at 37°C on tryptic soy agar (TSA) were then introduced into the TSBGlc and incubated for 24 hours. The cultures were subsequently transferred to a 96-well polystyrene microtiter plate with a flat bottom, following a 200-fold dilution with fresh TSBGlc. After 48 hours of incubation at 37°C, the planktonic cells were completely removed. The remaining biofilm was then washed three times for one minute each with 250 µL of phosphate-buffered saline (PBS) at a pH of 7.4. To stain the biofilm mass, a 0.1% (w/v) crystal violet solution was applied and left for 30 minutes at room temperature. Following three rounds of dye removal using 200 µL of PBS, the sample was allowed to air dry. The discoloured biofilm was mixed again in 200µ L of DMSO before to being quantified at a wavelength of 620 nm using a microplate reader. The citation is from the study conducted by Shin et al. in 2021 [30].

2.5. Antibiofilm Activity:

The bacterial cultures were diluted 200-fold in TSBGlc and separated into 100µL aliquots, which were then added to a 96-well polystyrene microtiter plate with a flat bottom. An aliquot of 100µL of Recipe G, produced in TSBGlc, was added to the 96-well microtiter plate. The effect of the examined medications on the biofilm mass of the pathogens was evaluated using the colorimetric technique following a 48-hour incubation at 37°C. The wells housing the media and substances under examination functioned as the control. The bacterial growth after administering these medications was also quantified using a

microplate reader in simultaneous trials. The citation "Straub et al, 2020" refers to a study conducted by Straub and colleagues in the year 2020 [31].

2.6. Phytochemicals:

The methanoli extracts were utilised for the subsequent assays, following the methodologies outlined by Chauhan et al, 2023 [32].

2.7.Quantitative determination of chemical constituency

The chemical composition levels (Coumarin, Tannic acid, and saponin) were obtained using the procedure described by ELBOUNY et al, 2022 [33].

2.8.Silver nanoparticles synthesis using polyherbal formulation:

6 ml of silver nitrate solution (10mM. 100mM, 150mM, 200mM, 250mM and 300mM) with 44 ml of extract. The reaction mixture was observed for color change depending on concentrations at room temperature, for 30 minutes at 80°C. The resultant reddish brown-colored reaction mixture was appeared, then the samples were transferred to centrifuge tubes and centrifuged at 12,000 rpm for 10 min. The pellet obtained was washed thrice with deionized water and finally with acetone. The resultant pellet was dried and stored for further characterizations [34].

2.9.Characterization of Silver nanoparticles:

UV analysis: 10mg/ml solution was prepared and then the absorbance was taken in the wavelength range 200 nm to 600 nm using UV-Vis spectrophotometer [35].

SEM: Morphology and structure of Nanoparticles was examined using scanning electron microscopy (SEM JSM-6360 (JEOL Inc. Japan) from the samples a small drop of Sample powder was sprinkled on SEM stub (pins) using double side adhesive tape and coated with aluminium at 20mA for 6minute through sputter-coater (Ion-Sputter JFC100). A scanning electron microscope with secondary detector will be used to obtain digital images of samples at an accelerating voltage of 15kV [35].

3. Results and Discussions

Figure 1 shows the extracted compounds of the leaves and seed of Pereskia aculeata, Sphagneticola trilobata, and Trachyspermum ammi collected in dark bottles. The solvent evaporation method is then used to carry out the extraction operation. An exhaustive and complete extraction of crude was accomplished by continuous iteration of the method, under room temperature, the resultant extract evaporated to a dry residue under lower pressure. The condensed residue was used for further research after being stored at 4 degrees Celsius.

Figure 1: Bioactive compounds are extracted from prepared ratios of materials.

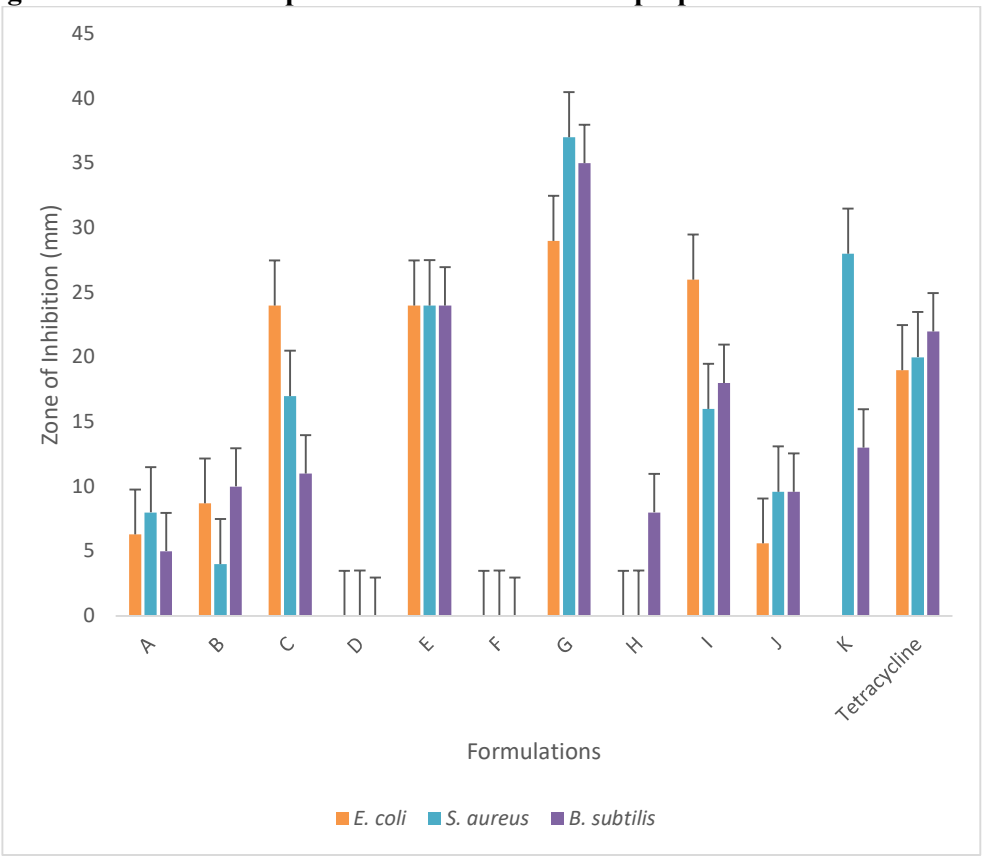

Figure 2: Antimicrobial analysis of prepared polyherbal recipes against three different pathogens.

Data obtained from this research elaborated that across all the polyherbal extracts, compared with antibiotics, polyherbal recipe G was very effective for four bacterial isolates *E. coli* (26±0.32mm), *S. aureus* (37±0.43 mm); *B. subtilis* (35±0.19 mm). Recipes D, F, and H did not show any zone of inhibition towards selected bacteria. Recipe K also did not show a zone of inhibition against E. coli. Recipe A showed a 6.3 ± 0.12 mm, 8±0.19 mm, and 5±0.16 mm zone of inhibition against *E. coli, S.aureus,* and *B. subtilis* respectively. Similarly for *E.coli* recipes, B, C, E, I, and J showed 8.7±0.32, 24±0.64, 24 ±0.67, 26 ±0.12, and 5.6±0.23 mm zone of inhibition. Furthermore, recipes, B, C, E, I, and J showed 4 ±0.32mm, 17±0.20, 24±0.16, 16±0.8, 9.6±0.10, and 28±0.48 mm, zone of inhibition respectively against *S. aureus*. Finally, recipes B, C, E, I, and J displayed the zone of inhibition 10±0.32,31±0.10, 24±0.30, 18±0.21, and 9.6±0.65 mm respectively against *B. subtilis.* These data were statically significant ($p<0.05$)

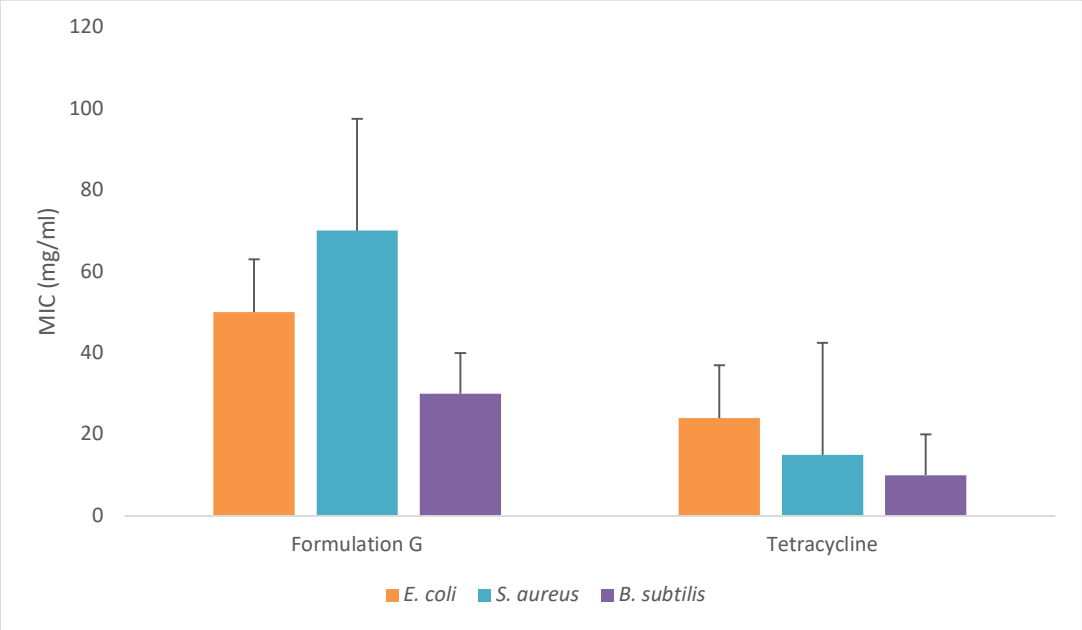

Figure 3: Graphical illustration of MIC studied against selected bacteria.

The minimum inhibitory concentration of formula G and tetracycline was assessed against specific microorganisms. The MIC values of recipe G against *E. coli, S. aureus*, and *B. subtilis* were determined to be 50 ± 0.8 mg, 70 ± 0.12 mg, and 30 ± 0.16 mg, respectively. The MIC values of Tetracycline against *E. coli, S.aureus*, and *B. subtilis* were 24 ±0.22mg, 15 ±0.10 mg, and 10±0.12 mg, respectively. The data showed statistical significance at $p<0.05$.

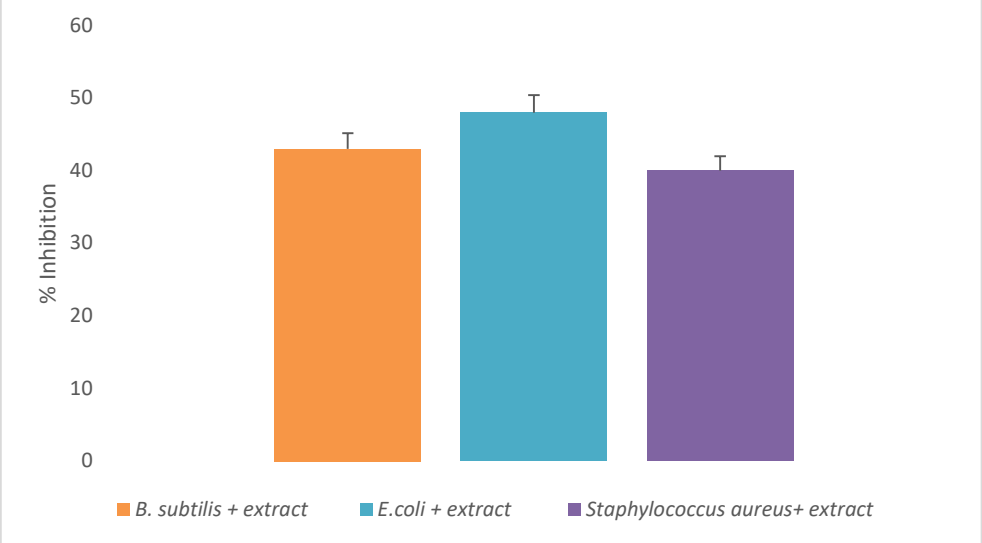

Figure 4: Evaluation of biofilm inhibition efficacy against pathogens.

Data showed that recipe G reduced biofilm development by 43±1.0%, 48±1.5%, and 40±2.0 % in *B. subtilis*, *E. coli,* and *S. aureus,* respectively. The data exhibited statistical significance with a p-value of less than 0.05.

Table 2: Analysis of phytochemical compounds present s in polyherbal extracts.

Acetone extract	**Name of the compound**
+	**Phenol**
+	**Saponins**

+	**Flavonoids**
+	**Tannins**
+	**Coumarin**
-	**Cardiac Glycoside**
+	**Alkaloid**
-	**Steroids**

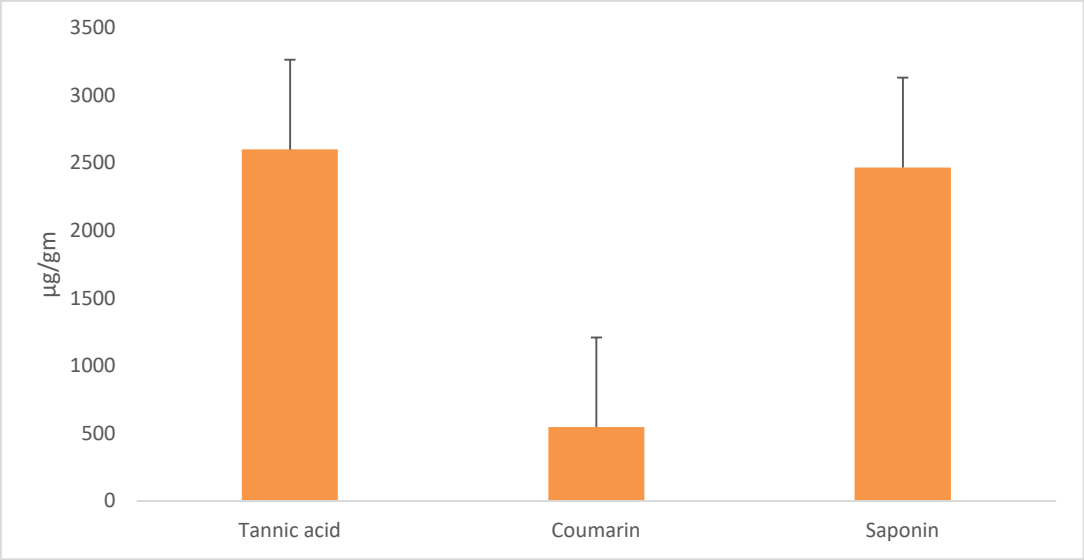

Figure 5: Quantitative determination of phytochemical substances in specimens. 2603±0.43 µg/gm of tannic acid, 547±2.04 µg/gm of coumarin, and 2470±2.10 µg/gm of saponin were found in the produced extracts of recipe G. The data showed a statistically significant relationship with a p-value below 0.05.

The emergence of antibiotic resistance presents a significant peril to human well-being. Several drugs lack efficacy in eradicating bacteria or treating wounds. The current study aims to discover the optimal ratio of several herbal combinations with potent antibacterial properties for effectively eradicating germs present in wounds. The assessment of several poly-herbal combinations in the present investigation unveiled synergistic, antagonistic, and additive interactions. The synergistic effect of poly-herbal formulations offers a pathway for the creation of effective antibiotics with different proportions of active components. Figure 2 presents data regarding the antibacterial activities of phytochemicals and plant extracts.

Four distinct components of the Poly-herbal plant *Pereskia aculeata*, *Trachyspermum ammi* leaves, *Trachyspermum ammi* seeds, and *Sphagneticola trilobata* leaves, were gathered. The water, hexane, acetone, and benzene extracts have demonstrated antibacterial action. The methanollic extract displayed the most significant zone of inhibition against all the strains tested, with a ratio of 1:1:1:2 (polyherbal recipe G) consisting of *Pereskia aculeata, Trachyspermum ammi* leaves, *Trachyspermum ammi* seed, and *Sphagneticola trilobata* leaves. Notably, it exhibited the highest activity against *S. aureus,* reaching up to 37 mm, as determined by the agar well diffusion method. Based on a comprehensive analysis of five years of research on antimicrobial properties and plant synergy, it has been found that the combined effect of plant extracts and antibiotics can be intensified through synergism [34].

The minimal inhibitory concentration was also assessed using a ratio of 1:1:1:2, consisting of *Pereskia aculeata, Trachyspermum ammi* leaves, *Trachyspermum ammi* seed, and *Sphagneticola trilobata.* The minimum inhibitory concentration (MIC) values against *E. coli, Staphylococcus aureus* (*S. aureus*), and *B. subtilis* were determined to be 50±0.26 mg/mL, 70±0.29 mg/mL, and 30±0.23 mg/mL, respectively (figure 3). Research conducted by Zhang et al. in 2022 [35] demonstrates that the combination of plants leads to a decrease in the minimum inhibitory concentration (MIC) against bacterial infections. Many plants include phytochemicals that have the ability to suppress many infections, including drug-resistant ones. The identification of phyto-compounds in plants is accomplished through qualitative analysis using phytochemical tests, as described by Sownthariya et al. (2022) [36]. Furthermore, medicinal plants possess a substantial amount of secondary metabolites and have the potential to serve as valuable reservoirs of pharmaceutical compounds. The secondary metabolites encompass alkaloids, glycosides, coumarins, flavonoids, steroids, and other compounds. Plants are a significant reservoir of diverse natural products, which vary greatly in their structures, biological features, and mode of action [37].

Multiple studies have shown that the ability of *Staphylococcus aureus, B. subtilis,* and *E.coli* to create biofilms is considered an important characteristic that enhances the pathogen's ability to survive in the udder, evade the host's immune system, and impede antibiotic treatment. The presence of biofilm in chronic wounds hampers their ability to heal by producing harmful enzymes and poisons that contribute to a persistent inflammatory condition within the wound [38]. The capacity of these three isolates to generate biofilms was seen in Figure 6. Therefore, it was chosen to evaluate the effectiveness of recipe G in inhibiting biofilm formation. Their impact on the prevention of biofilm formations. The recipe G exhibited comparable inhibitory effects on the biofilm development of both *Staphylococcus aureus* (S. aureus) and *B. subtilis* (B.s).

Further the polyherbal formulation was used for the synthesis of nanoparticles and using silver nitrate as a salt. The synthesized nanoparticles was then screened for the characterization by using UV analysis and SEM for size identification. The results revealed that the nanoparticle synthesized shows maximum absorbance at 410 nm as shown in figure 6. Similarly, Ahmed et al., 2021 synthesized silver nanoparticles and found 416 nm in UV spectral analysis [39].

AgNPs-PE demonstrated spotted nanoparticles with more homogeneous and non-aggregated particles in the current investigation (Figure 7). AgNPsPE showed up as amorphous, spherical, and comparatively bigger particles [40].

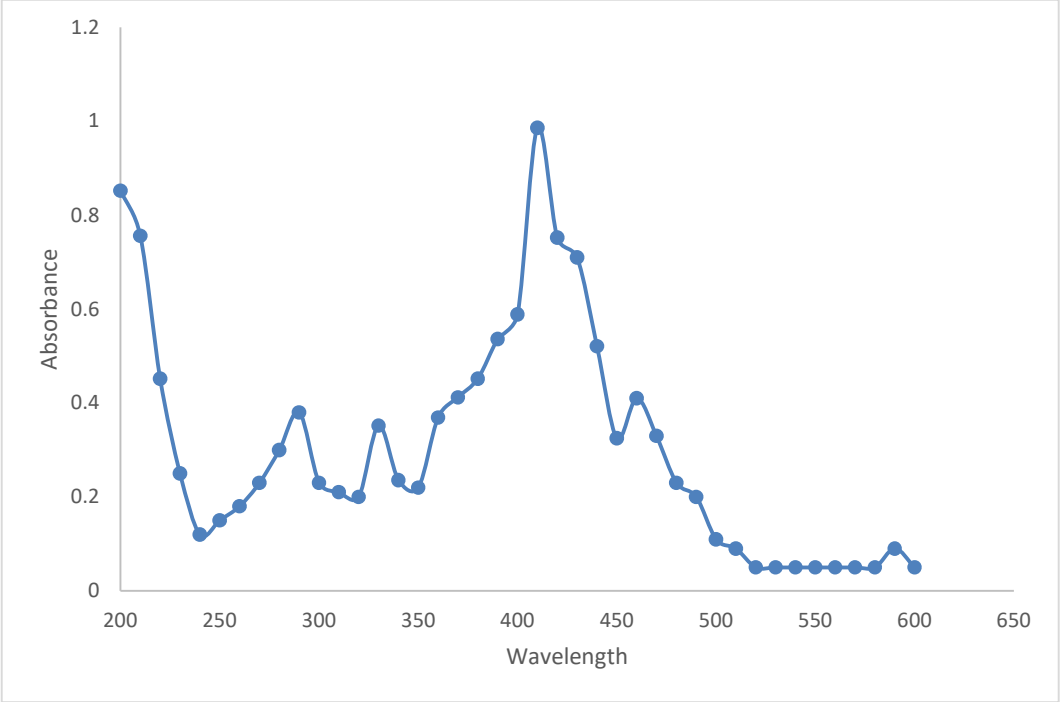

Figure 6: UV analysis of the synthesized silver nanoparticles using polyherbal extract.

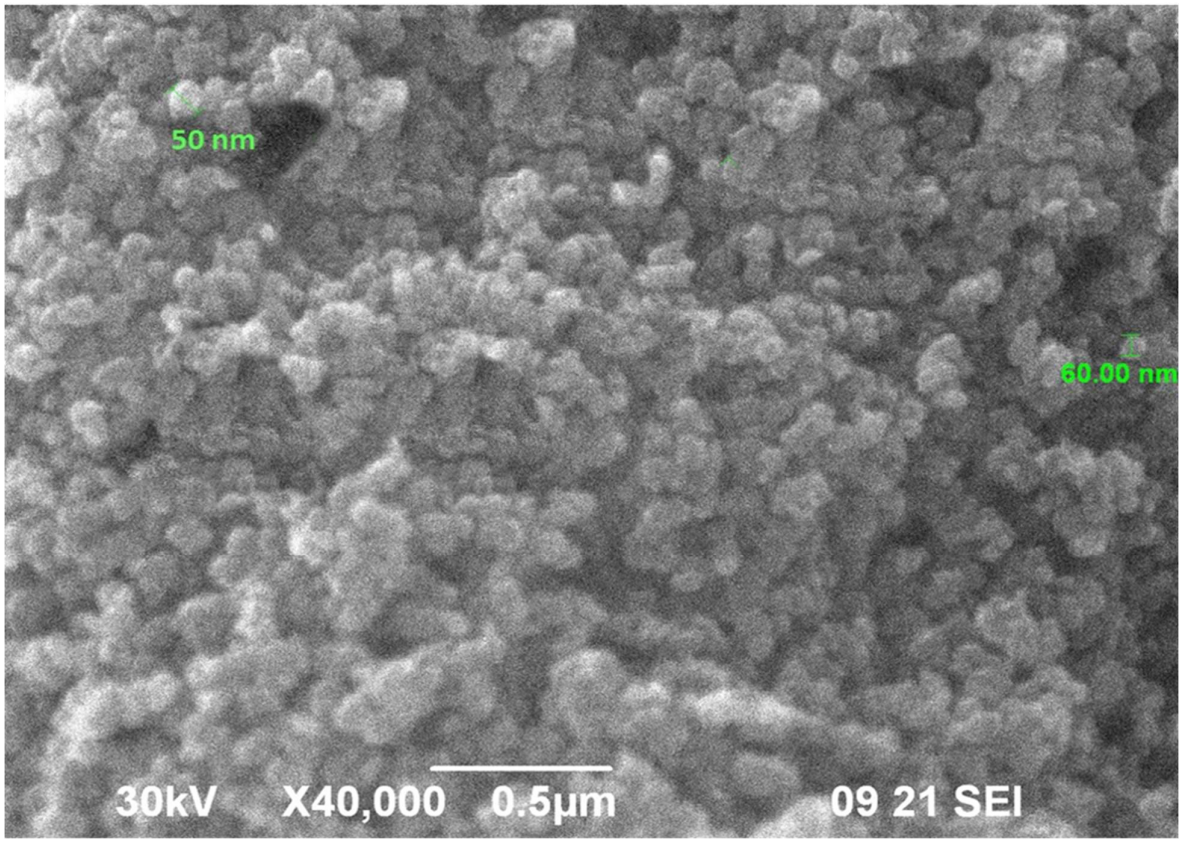

Figure 7: SEM analysis of the synthesized silver nanoparticles using polyherbal extract.

4. Conclusion

Polyherbal is an abundant reservoir of phytochemicals, encompassing a wide array of substances including, terpenoids, flavonoids, tannins, steroids, phenolic compounds, alkaloids, coumarins, carbohydrates, proteins, anthraquinones, quinines, and saponins. The findings of this investigation revealed the extensive range of antimicrobial properties exhibited by the extract. Furthermore, the findings demonstrated that the extract exhibited greater efficacy as an anti-biofilm agent. The actions seen may be attributed to the presence of phenolic compounds, such as saponin, coumarin, and tannic acid, which operate as the primary active ingredients.

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